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Genotype and functional correlates of disease phenotype in deficiency of adenosine deaminase 2 (DADA2)

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PYL, PAN and QZ designed the study. PYL, ESK, EF, CDP, TS, SR, IT, AW, MBJ, CK, DE, PB, AS, ZB, GL, CI, SSK, RE, ML, PP, RK, ECC, JC, RG and QZ contributed clinical data. PYL, YH, WB, MFA, and KS performed experiments. PYL, ESK, YH, ZH, PAN and QZ analyzed the data. PYL, PAN and QZ drafted the manuscript and all authors revised the manuscript.

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Abstract

Background—Deficiency of adenosine deaminase 2 (DADA2) is a syndrome with pleiotropic manifestations including vasculitis and hematologic compromise. A systematic definition of the relationship between *ADA2* mutations and clinical phenotype remains unavailable.

Objective—We tested whether the impact of *ADA2* mutations on enzyme function correlates with clinical presentation.

Methods—DADA2 patients with severe hematologic manifestations were compared with vasculitis-predominant patients. Enzymatic activity was assessed using expression constructs reflecting all 53 missense, nonsense, insertion and deletion genotypes from 152 patients across the DADA2 spectrum.

Results—We identified DADA2 patients presenting with pure red cell aplasia (PRCA, n = 5) or bone marrow failure syndrome (BMF, n = 10). Most patients did not exhibit features of vasculitis. Recurrent infection, hepatosplenomegaly and gingivitis were common in patients with BMF, of whom half died from infection. Unlike DADA2 patients with vasculitis, patients with PRCA and BMF proved largely refractory to tumor necrosis factor inhibitors. *ADA2* variants associated with vasculitis predominantly reflected missense mutations with at least 3% residual enzymatic activity. By contrast, PRCA and BMF were associated with missense mutations with minimal residual

enzyme activity, nonsense variants, and insertions / deletions resulting in complete loss of function.

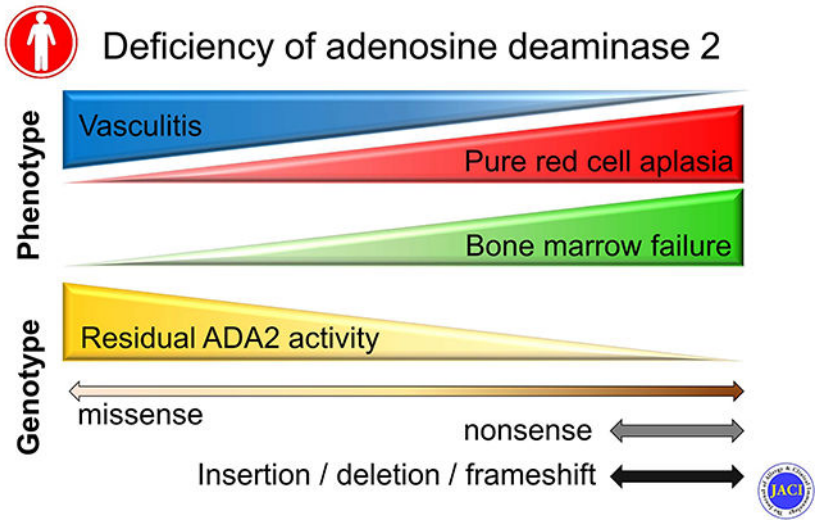
Conclusion—Functional interrogation of *ADA2* mutations reveals an association of subtotal function loss with vasculitis, typically responsive to TNF blockade, whereas more extensive loss is observed in hematologic disease which may be refractory to treatment. These findings establish a genotype-phenotype spectrum in DADA2.

Clinical implications—Genotype correlates with clinical phenotype and therapeutic response in DADA2.

Capsule Summary

DADA2 is a monogenic disorder with multi-organ system manifestations. We present a cohort of DADA2 patients with severe hematologic defects and describe novel genotype-phenotype correlations based on functional analysis of 53 *ADA2* mutations.

Graphical Abstract



Keywords

adenosine deaminase 2; DADA2; vasculitis; pure red cell aplasia; bone marrow failure

Introduction

Deficiency of adenosine deaminase 2 (DADA2) is a monogenic autoinflammatory disease initially characterized as a cause of stroke and systemic vasculitis in young children [1, 2]. Since its initial description in 2014, the clinical spectrum of this condition has expanded considerably, and variable hematologic and immunologic abnormalities have been described in about half of DADA2 patients [3, 4] Primary presentations of the disease include pure red cell aplasia (PRCA) that mimics Diamond-Blackfan anemia and bone marrow failure (BMF) with variable cytopenia, even without vasculitis or systemic inflammation [5–7]. The severity of these manifestations can result in transfusion dependency in patients with PRCA or a

need for hematopoietic stem transplant (HSCT) in those with BMF [8–11]. Some patients present with humoral immunodeficiency and recurrent infection, further complicating our understanding of DADA2 [12, 13]. How mutations in the same gene can present with different phenotypes is poorly understood.

ADA2 is an extracellular enzyme primarily secreted by monocytes and macrophages [14, 15]. While ADA2 is capable of catalyzing the deaminase reaction that converts adenosine to inosine, its physiologic function is not known. Biallelic mutations in the encoding gene *ADA2* (formerly known as *CECR1)* and very low levels of ADA2 enzymatic activity in the peripheral blood are diagnostic of DADA2 [2]. Missense variants are most common but nonsense mutations, insertions / deletions (indels) and splice site mutations have been described [4]

A systematic analysis comparing *ADA2* mutations associated with different clinical phenotypes is lacking. Previous studies have not been able to establish convincing genotype-phenotype correlations, in part due to a limited number of cases and preferential recruitment of patients with a specific phenotype based on the subspecialty of the investigators. Establishing genotype-phenotype correlations has important diagnostic and therapeutic implications. Whereas tumor necrosis factor inhibitors (TNFi) prevent strokes and improve manifestations of vasculitis in DADA2 [16], their efficacy for PRCA and BMF is less clear. HSCT may be considered earlier for patients with severe hematologic presentations [9].

Here we report 15 new cases of DADA2 with PRCA or BMF as primary presentation. Based on the genetic findings we observed in these patients, we systematically studied *ADA2* mutations from 152 published cases encompassing the different phenotypes by in silico analysis and functional assay. Our results provide strong evidence for genotype-phenotype correlations in DADA2 with potentially direct clinical relevance.

Methods

Patients

These studies were approved by the Institutional Review Boards at Boston Children's Hospital and Brigham and Women's Hospital. We performed retrospective chart review of 15 patients with DADA2 from 12 families. The patients were enrolled through a world-wide collaboration with approval by the local ethics committees. Research diagnostic testing was performed with written informed consent from the parent or guardian and assent when appropriate. Clinical and laboratory data for the cohort are described in Tables E1–E3 in Online Repository).

Literature search

Please see Supplemental methods in Online Repository for details of literature review and criteria for case selection. Cases selected from each publication and their phenotype are detailed in Table E4 in Online Repository. A complete list of mutations from the selected cases are displayed in Table E5 in Online Repository.

Analysis of *ADA2* mutations

Construction of pcDNA3.1 plasmid for expression of wild-type ADA2 was as described [17]. Site-directed mutagenesis was performed using the NEB Q5 mutagenesis kit (New England Biolabs, Ipswich, MA). The list of mutations and primer pairs used to generate mutant constructs are available in Tables E6 in Online Repository. Mutant constructs were purified using Purelink Quick Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA) and verified by sequencing. Plasmids were transfected into 293T cells using Fugene 6 (Promega, Madison, WI). Medium was collected after 72 hours and ADA2 activity was quantified using an established spectrophotometric assay that couples the release of ammonia from adenosine with the consumption of NADH [217]. Each mutant was analyzed by three independent experiments and measurements were normalized to the activity of wildtype ADA2 from the same run.

Statistical analysis

The Kruskal-Wallis test was used for comparison of ADA2 activity between multiple mutation types and disease phenotypes. Chi-square was used for comparison of mutation types between clinical phenotypes. All tests were two-sided, and $P < 0.05$ was considered significant. Statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA).

Results

A series of DADA2 patients with primary hematologic defects

We present an international cohort of 15 DADA2 patients from 12 families with PRCA (n = 5) or BMF (n = 10) as their primary presentation. Summarized data for the cohort are displayed in Table 1. Clinical manifestations and laboratory data for each patient are provided in Tables E1 and E2 in Online Repository, respectively. The age of onset for PRCA was very early (median 0.3 years, range 0.1 – 12 years); only 1 patient presented after 6 months of age (Table 1). The age of onset was more variable for the BMF group (median 2.2 years, range 0.1 – 13 years). Patients with PRCA displayed normocytic or microcytic anemia with very low reticulocyte count, consistent with defective erythrocyte production. Most patients with BMF had severe neutropenia and mild anemia, while 2 patients had pancytopenia. Consistent with previous studies, low immunoglobulin levels (IgG, IgM and/or IgA) were common in patients with DADA2 (Table 1). Cases of severe infection have been described in DADA2-associated BMF [8, 18, 19]. Indeed, recurrent infection was more common in the BMF group (80% vs 20% in PRCA group). These patients experienced a variety of infections, and 5/10 patients ultimately succumbed to sepsis (Table E1 in Online Repository). It is noteworthy that two patients (K-1 and L-1) each had one sibling that died from severe infection before the discovery of DADA2, suggesting that mortality for this phenotype is even higher than estimated here.

Unlike patients from previous large series focused on DADA2 as a monogenic vasculitis [1, 2, 20–22], most DADA2 patients in this PRCA / BMF cohort (12/15, 80%) had no history of vasculitis. Two patients with BMF had cutaneous vasculitis and one patient with PRCA developed sudden-onset squinting and transient hemiparesis with MRI findings compatible

with a small ischemic stroke. In the BMF group, almost all patients exhibited hepatosplenomegaly and half experienced severe gingivitis, a feature associated with neutropenia [23] that has not previously been reported in DADA2 (Table E1 in Online Repository). Treatment regimens for these patients include disease modifying anti-rheumatic drugs (DMARDs), biologics, intravenous immunoglobulin, granulocyte colony stimulating factor (GCSF) and HSCT. Unlike the success of TNFi therapy for prevention of stroke and treatment of vasculitis [16], most cases in this cohort did not respond to TNFi (Table E1 in Online Repository). In 10 patients that received TNFi, only one (patient C-1 with PRCA and stroke) showed sustained improvement of hematologic features. Three patients showed improvement of vasculitis and/or systemic inflammation but their cytopenia did not improve. One patient with BMF developed *Pseudomonas aeruginosa* sepsis soon after initiation of TNFi.

Patients in this cohort did not exhibit clinical features of autoimmunity. All patients with PRCA showed negative direct Coomb's test. Four patients in the BMF group were found to have autoantibodies: 2 with low-titer anti-nuclear antibodies, 1 with anti-neutrophil antibodies, and 1 with non-specific anti-neutrophil cytoplasmic antibodies (Table E3 in Online Repository).

Biallelic mutations in *ADA2* were confirmed in all patients (Table E1 in Online Repository). Nine unique *ADA2* mutations were found in this cohort, and two (F212del and K449Nfs*2) were novel variants (Table 2). To confirm the pathogenicity of these mutations, we expressed these variants in 293T cells and measured ADA2 activity using an established spectrophotometric assay [2,24] All mutations from our patient cohort displayed minimal residual ADA2 activity (<2% of wildtype; Table 2). Interestingly, among the 7 previously-described mutations, 6 had been described in patients with severe hematologic manifestations without vasculitis [6, 8, 10, 13, 18, 25, 26]. Moreover, whereas most vasculitis-associated *ADA2* mutations in prior studies were missense variants [4], 6 patients in this cohort had homozygous indel mutations resulting in frameshift and early truncation. All siblings in one family (A-1, A-2 and A-3) exhibited PRCA while another pair of siblings had severe neutropenia (J1 and J2). These observations together raised the possibility of genotype differences among the clinical phenotypes in DADA2.

Genotype comparison of vasculitis and hematologic phenotypes in DADA2

To investigate possible genotype-phenotype correlations, we performed a literature review of published DADA2 cases with vasculitis, PRCA or BMF as the primary presentation. A list of included studies and details of case selection are provided in Methods and Table E4 in Online Repository. We reviewed 186 cases, of which 152 were selected for further investigation (Figure 1A). Details of case selection and exclusion are provided in Supplemental Method in Online Repository. Cases that appeared in multiple publications were analyzed only once and those with other phenotypes or incomplete data on *ADA2* mutations were excluded (Table E4 in Online Repository). Because vasculitis is the most common presentation of DADA2, the vasculitis group (n = 100) was pooled from 11 major case series from around the world to minimize bias and regional differences. Including the 5 cases in our cohort, we identified 38 cases of DADA2 with PRCA as the primary

manifestation. The BMF group consisted of 29 cases including the 10 patients from our cohort. Two patients with PRCA and 5 in the BMF group were described to have features of vasculitis.

One notable demographic difference between groups was age at presentation. In line with the observation in our cohort, DADA2 patients with PRCA presented very early in childhood [median age 0.5 years; interquartile range (IQR) 0.2 – 2.6] while those with vasculitis and BMF generally presented later (vasculitis: median 5.0 years, IQR 1.0 – 10.0 vs. BMF: median 5.0 years, IQR 2.0 – 14.0), including many cases diagnosed in adulthood (Kruskal-Wallis test, $p < 0.001$; Figure E1 in Online Repository).

Among the three groups (167 combined patients), 61 unique *ADA2* mutations were found (Figure 1B). Surprisingly, only two of these mutations were shared by all three groups: H112Q and R169Q. The greatest overlap was found between PRCA and BMF groups, with 7 shared mutations not reported in the vasculitis group. Two mutations were shared by the vasculitis and PRCA groups, while another two were shared by the vasculitis and BMF groups. Plotting *ADA2* mutations according to exon location, mutations associated with all three groups were scattered throughout the gene, without preferential concentration in specific domains (Figure 1C).

When the types of mutation were characterized (counting each allele individually), most mutations associated with vasculitis were missense variants (Figure 1D). Less than 10% of the mutant alleles in the vasculitis group belonged to other categories. In contrast, missense mutations accounted only for 53% of the variants in the PRCA group and 72% in the BMF group, respectively (Chi-square, $p < 0.0001$). Indels comprised the majority of remaining mutations for both groups (38% for PRCA and 16% for BMF; Figure 1D). When cases with compound heterozygous mutations were excluded, all 63 patients in the vasculitis group had homozygous missense mutations while more variable mutation types were found in the PRCA and BMF groups (Chi-square, $p < 0.0001$).

Using histograms to assess the most common *ADA2* variants in each group, only a few overlapping mutations were found between the groups. R169Q was found in multiple patients in all three groups, while the most common mutation associated with vasculitis, G47R, was not seen in the other groups (Figure 2B). In contrast, G358R was seen in patients with PRCA and BMF, but not in those with vasculitis. R169Q and G358R were the only variants in the BMF group found in more than 2 cases.

Functional analysis of *ADA2* mutations

The abundance of indels in the PRCA group suggests that the more detrimental mutations may be associated with this phenotype. However, missense mutations still accounted for more than 50% of variants. To understand whether functional differences exist among mutations groups, we created expression plasmids for each *ADA2* mutant and transfected them into 293T cells. ADA2 enzymatic activity in the supernatant served as a functional readout for each mutation. Constructs for all 53 missense, nonsense, and indel variants from our patient cohort and published cases (Table E5 in Online Repository) were analyzed using

this method. Splicing defects were not evaluated as the sequences for aberrantly-spliced complementary DNA are not available.

Our functional analysis confirmed that all mutations caused a reduction in ADA2 activity (Figure 3A). Not surprisingly, early translational termination caused by nonsense mutations and indels with frameshift completely abrogated ADA2 function. Missense variants, on the other hand, showed a wide spectrum of impact ranging from partial to complete loss of enzyme activity. Stratification by patient phenotype showed significantly greater residual ADA2 activity for mutations associated with vasculitis compared those associated with PRCA or BMF (Kruskal-Wallis test, $p = 0.0002$; Figure 3B). Examination of different cut-off levels revealed that a residual activity of >3% effectively segregated half of mutations associated with vasculitis from the other two groups (Chi-square, $p < 0.0001$; Figure 3C,D). All mutations associated with PRCA or BMF displayed residual activity under this threshold aside for Y353H, which demonstrated 4% residual activity.

To ensure that the statistics were not skewed by the greater number of nonsense and indel variants in the PRCA and BMF groups, we repeated the analysis including only missense mutations. A similar pattern was observed, as missense *ADA2* mutations associated with vasculitis displayed significantly more residual enzymatic activity than those associated with the hematologic phenotypes (Kruskal-Wallis $p < 0.0001$; Figure E2A in Online Repository). We applied several in silico prediction algorithms to assess the pathogenicity of missense mutations associated with the three phenotypes. Consistent with our experimental data, analysis by SIFT [27] predicted that mutations associated with vasculitis would impair gene function significantly less than those associated with PRCA or BMF (Figure E2B in Online Repository). Such difference was not predicted by other algorithms (Polyphen2, MutationTaster and CADD; Figure E2C–F in Online Repository). Taken together, these findings suggest that the more deleterious *ADA2* mutations are associated with severe hematologic phenotypes.

Establishing genotype-phenotype correlations in DADA2

Analysis of individual mutations cannot account for patients with compound heterozygous mutations. To evaluate whether the functional studies can be utilized to predict phenotype using actual mutation configurations from patients, we clustered *ADA2* mutations into three categories using the 3% residual activity cut-off: type A, hypomorphic missense variants with ≥ 3% residual enzymatic activity compared to wildtype ADA2; type B, missense mutations with minimal (<3%) residual activity, and type C, indels and nonsense mutations with complete absence of enzyme activity. Based on the biallelic mutations identified, each patient was assigned to one of 6 groups (AA, AB, AC, BB, BC, CC) that reflected the predicted functional category of both mutations. For example, a patient with two type A mutations was assigned to group AA, while another patient with compound heterozygous type B and type C mutations was assigned to group BC. This method stratifies biallelic mutations for each patient into groups with a gradient of predicted residual ADA2 activity, where groups AA and CC have the highest and the lowest predicted activity, respectively. Patients with splice-site mutations (n = 7) were excluded from this analysis due to the lack of functional data to evaluate these variants.

For each phenotype, the percentage of patients assigned to each mutation group was plotted. Almost all DADA2 patients with vasculitis had at least one mutation with ≥ 3% residual ADA2 function and therefore were distributed to the AA, AB and AC groups (Figure 4). Most of the remaining patients, the majority of whom were homozygous for R169Q, were assigned to the BB group. By contrast, the majority of PRCA and BMF cases were found in the BB, BC, and CC groups, which have lower predicted residual ADA2 function (Chi-square, $p < 0.0001$). To reflect the actual number of cases in each category, all three groups were plotted together in Figure E3 in Online Repository. Accordingly, the prevalence of BMF and PRCA cases was greater in the genotype categories predicted to have lower residual ADA2 activity. These findings support the existence of genotype-phenotype correlations in DADA2, where missense mutations with greater residual enzymatic function favor the development of vasculitis while more detrimental missense mutations, indels and early-termination mutations causing more extensive disruption of protein function are associated with hematologic manifestations.

Discussion

DADA2 was first described as a form of monogenic vasculitis that mimics polyarteritis nodosa. Case reports and small case series have subsequently established severe hematologic defects as an alternate presentation of DADA2. The 15 new cases with PRCA / BMF described in this study represent the largest series to date for the severe hematologic phenotype of DADA2. We found that patients with PRCA tend to present very early in life and that those with BMF exhibit a high rate of mortality from recurrent infections. Extending the functional analysis of *ADA2* mutations to include the more than 150 published cases in the literature, our work provides new evidence for genotype-phenotype correlations in DADA2. Mutations that are most detrimental to protein function, as measured by residual ADA2 activity, are enriched in patients with severe hematologic involvement.

Genotype-phenotype correlations in DADA2 have been difficult to establish due to incomplete penetrance and variable clinical manifestations in family members with identical mutations [21]. Further, large case series have primarily characterized patients with vasculitis. A recent study comparing 12 patients with vasculitis and 10 with PRCA concluded that mutations in the dimerization domain of ADA2 were associated with vasculitis while those in the catalytic domain aligned with PRCA [11]. However, almost all patients with vasculitis shared the G47R missense mutation. Our analysis of a broader range of variants showed that mutations associated with each phenotype were distributed throughout the gene without preferential location to specific domains.

The physiologic function of ADA2 is not fully elucidated and therefore it remains unclear whether the same mechanism underlies the development of vasculitis and hematologic defects. Based on our stratification of *ADA2* mutations, it is possible that only a small amount of ADA2 is required to maintain normal hematopoiesis, since patients with the most detrimental mutations are most prone to developing PRCA and BMF. It remains unexplained why identical mutations result in PRCA in some patients and BMF in others. Whether ADA2 is produced by specific hematopoietic progenitor cells or acts differentially on these

cells as a soluble factor warrants further investigation. Additional modifier genes and extrinsic factors in the bone marrow environment may also contribute to variable phenotype.

How *ADA2* mutations with residual enzymatic function cause vasculitis remains to be determined. Carmona-Rivera et al. recently found that in the absence of ADA2, adenosine can trigger the formation of neutrophil extracellular traps (NET), which stimulates macrophages to produce TNF-α [28]. While this mechanism may explain the basis of vasculitis and the effectiveness of TNFi therapy, it does not account for the observation that patients with the most detrimental mutations (e.g. nonsense and indels with frameshift) often do not present with vasculitis. It remains to be seen whether the same mechanism applies to the hematologic phenotype of DADA2, which seems less responsive to TNFi. Of the 10 patients that received TNFi in this case series, only one patient showed sustained improvement of PRCA after initiation of etanercept and corticosteroids. Three other patients showed improved fever and/or systemic inflammation without amelioration of baseline cytopenia. Recurrent infections are common in patients with BMF, and the addition of TNFi may further compromise immune defense. Additional studies are needed to address whether TNFi should be used routinely for all DADA2 patients.

Critically, the amount of ADA2 generated by our overexpression system is 20-fold higher than the average plasma ADA2 activity (>250 U/L for supernatant from 293T cells, compared to 12 U/L for healthy adult control plasma) [15]. This overexpression increases the dynamic range of our measurements, enabling us to demonstrate that *ADA2* mutations associated with vasculitis provide greater residual function than mutations associated with PRCA and BMF. In clinical practice, measurement of plasma ADA2 is limited by the inability of current techniques to resolve small differences in residual activity, which in most DADA2 patients therefore appears uniformly very low. We do not expect that the difference between levels of residual ADA2 function confirmed here in vitro will be observable in clinical samples, as would be required to extrapolate an expected clinical course from measured plasma ADA2 activity.

Although we here stratified each case into a phenotypic category based on primary manifestations, DADA2 phenotypes likely represent continua rather than distinct categories. DADA2 patients with vasculitis can develop anemia and leukopenia as part of their clinical course. Similarly, patients with severe hematologic defects remain susceptible to vasculitis. This potential overlap in phenotypic spectra is best illustrated by the R169Q missense variant, one of the most common pathogenic mutations in DADA2. The R169Q substitution renders minimal residual ADA2 activity (category B, <3%) and is found in all phenotypic categories in our analysis. Supporting this view, a wide spectrum of manifestation including stroke, red cell aplasia and profound cytopenias were reported in a cohort of patients with homozygous R169Q mutations [29]. Inference from genotype is also complicated by high prevalence of compound heterozygosity in DADA2. Therefore, our ability to predict phenotype based on genotype alone remains limited and treatment decisions should be guided by clinical findings.

The spectrum of clinical manifestations in DADA2 extends beyond vasculitis, PRCA and BMF. Common variable immunodeficiency (CVID) has been recently described as a

manifestation of the disease [13]. It is unclear whether CVID represents a distinct phenotype in DADA2 because many of these patients also exhibited vasculitis and hematologic defects. The prevalence of hypogammaglobinemia in our cohort is similar to the general estimate for all patients [3]. We suspect that humoral immunodeficiency with low immunoglobulin levels likely represents a common clinical feature of DADA2 regardless of the presenting phenotype. DADA2 can also manifest as autoimmunity (systemic lupus erythematosus and anti-phospholipid syndrome) and lymphoproliferative disease [30, 31]. Patients in our cohort did not exhibit these features and autoantibodies were present only in a few patients. With only a limited number of cases reported, it is difficult to establish genotype correlations for these uncommon DADA2 phenotypes.

The broad clinical spectrum of DADA2 and variability in patient presentation are well recognized, but little is known about factors that influence disease phenotype. By characterizing a cohort of patients with PRCA or BMF, our work highlights the severity of hematologic manifestations and their associated morbidity and mortality in DADA2 patients. Systematic comparison of *ADA2* mutations in patients with vasculitis, PRCA and BMF through functional analysis revealed a distinct correlation between mutation pathogenicity and disease phenotype. Further studies are needed to determine differences in the underlying pathophysiology of vasculitis and hematologic defects in DADA2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADA2	adenosine deaminase 2
DADA2	Deficiency of ADA2
BMF	Bone marrow failure
CADD	Combined Annotation Dependent Depletion
CVID	combined variable immunodeficiency
GCSF	granulocyte colony stimulating factor
HSCT	Hematopoietic stem cell transplant

PRCA	Pure red cell aplasia
TNF	Tumor necrosis factor
TNFi	TNF inhibitor

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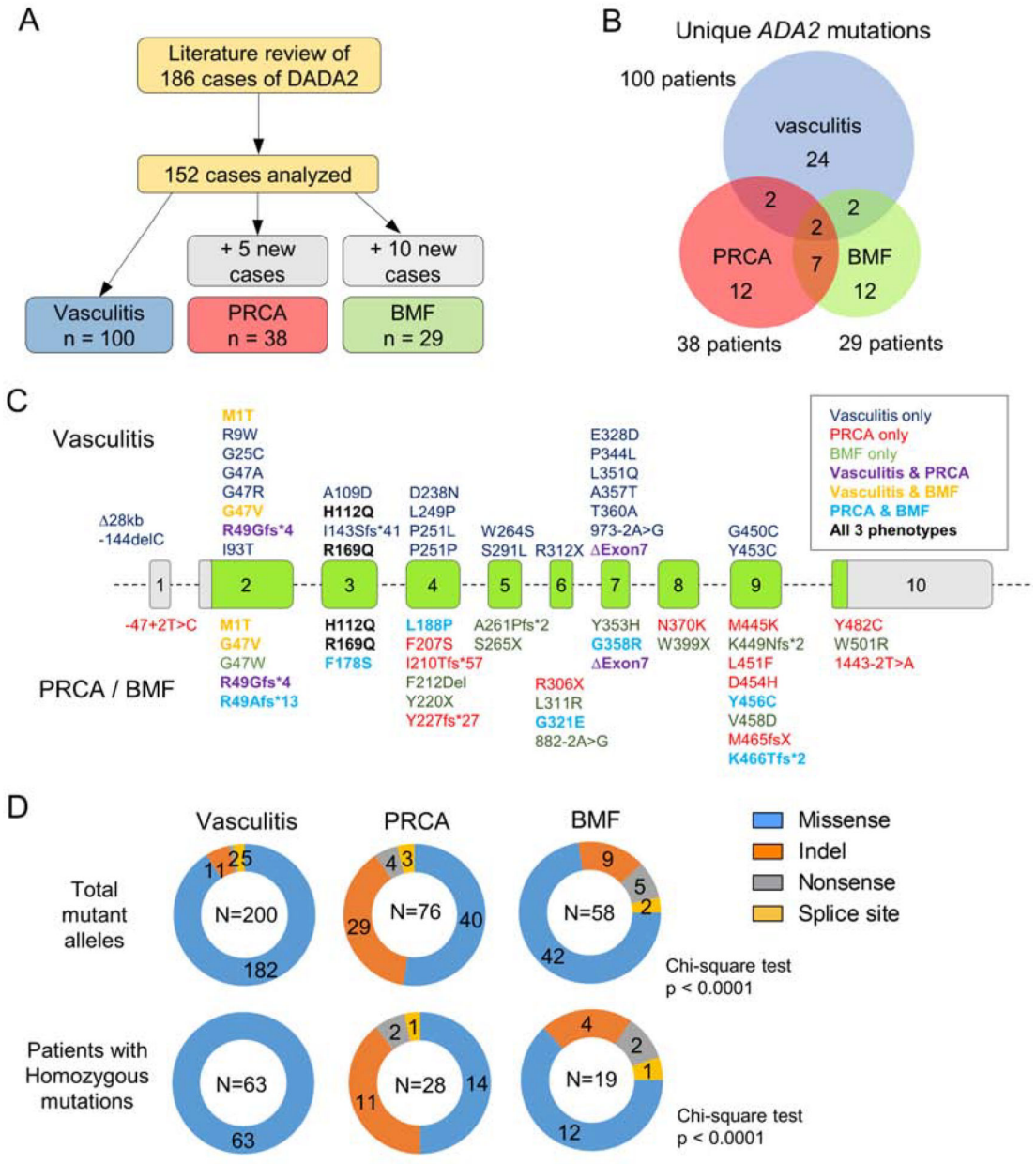


Figure 1. Analysis of *ADA2* mutations by patient phenotype.

A) Schematic of literature review and case selection for mutation analysis. B) Venn diagram of unique *ADA2* mutations illustrating overlaps between disease phenotypes. C) Display of *ADA2* gene structure illustrating the distribution of mutations associated with different phenotypes. Shared mutations are displayed by color coding. D) Circle charts illustrating the types of mutations associated with each phenotype. Analysis of individual alleles is displayed in the upper panel while analysis of homozygous individuals is shown in the lower panel.

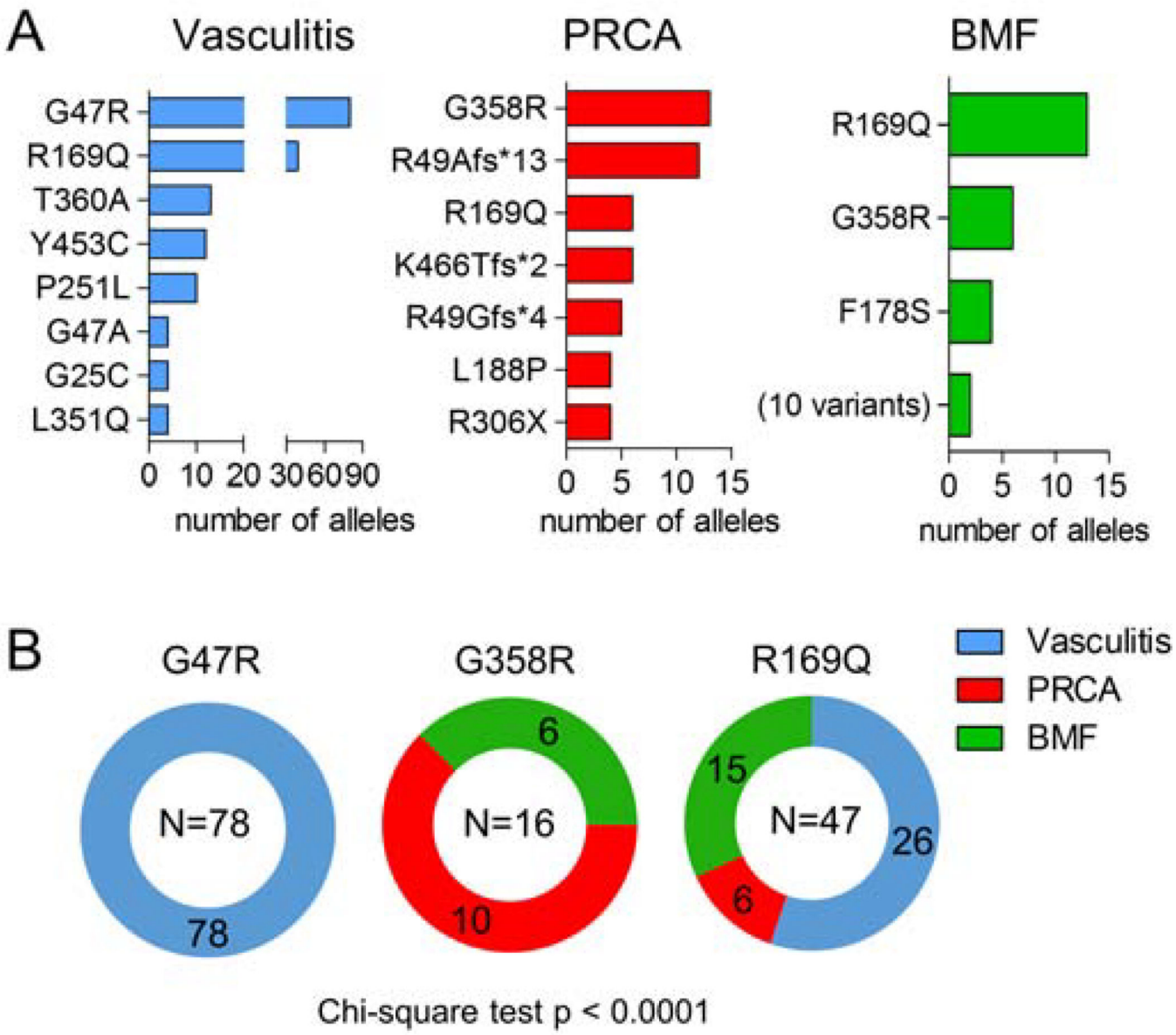
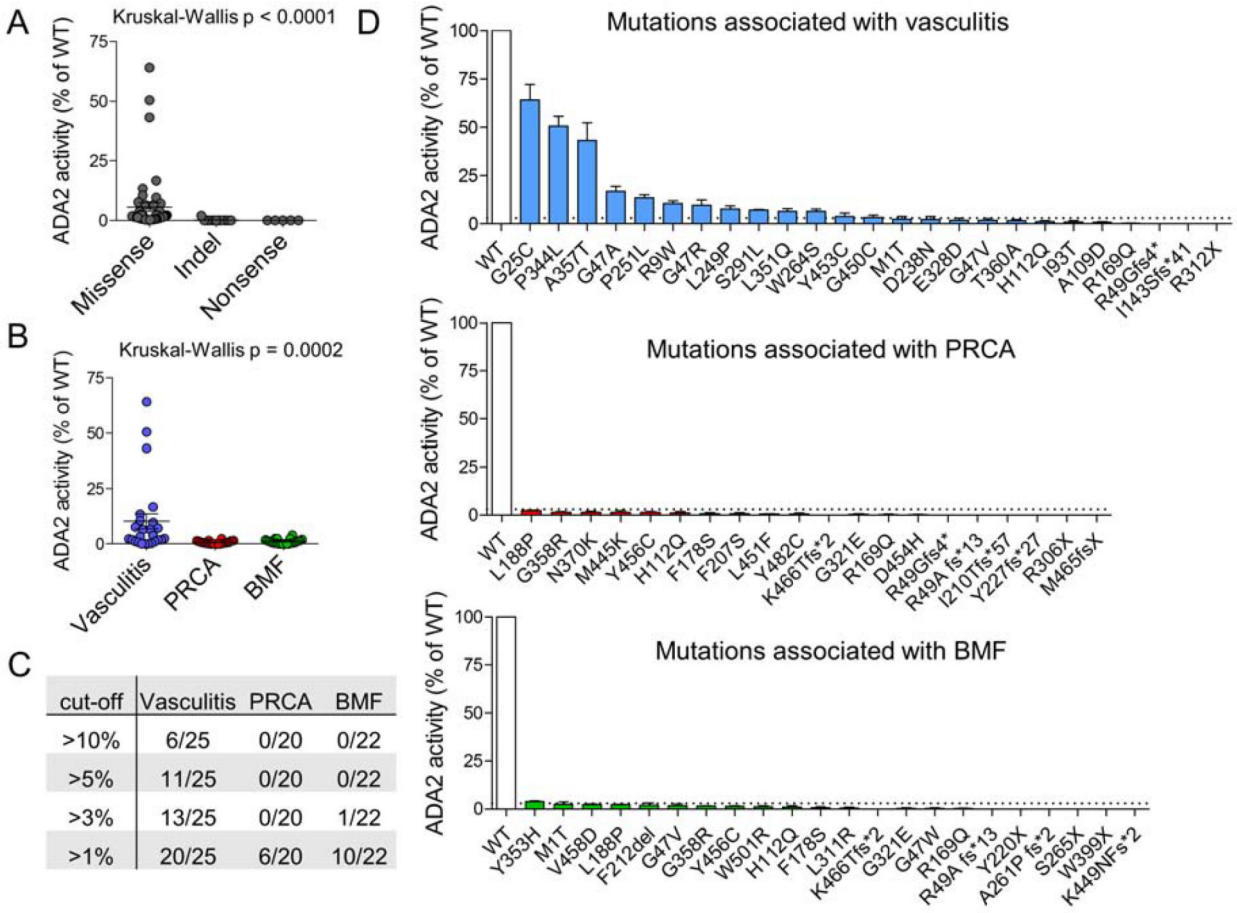


Figure 2. Analysis of common mutations associated with each disease phenotype.

A) Histogram display of allelic count for the most common mutations associated with each disease phenotype. All cases in the current cohort and those selected from literature review were included. B) Phenotype distribution of the most common mutation association with vasculitis (G47R), PRCA (G358R) and BMF (R169Q).



cut-off	Vasculitis	PRCA	BMF
>10%	6/25	0/20	0/22
>5%	11/25	0/20	0/22
>3%	13/25	0/20	1/22
>1%	20/25	6/20	10/22

Figure 3. Functional analysis of *ADA2* mutations in vitro.

A) ADA2 enzyme activity of individual mutant constructs sorted by mutation type. B) ADA2 enzyme activity of individual mutant constructs sorted by disease phenotype. C) Stratification of mutations within each disease phenotype according to various cut-off values of residual ADA2 enzyme activity. D) Bar graph display of residual enzyme activity for individual mutations associated with each disease phenotype. Dotted line in all panels represent the cut-off value of 3% residual activity. For all panels, results are normalized as percentage of residual activity relative to wildtype (WT) ADA2. Each dot or bar represents the average of results from three independent experiments and error bar represents standard deviation.

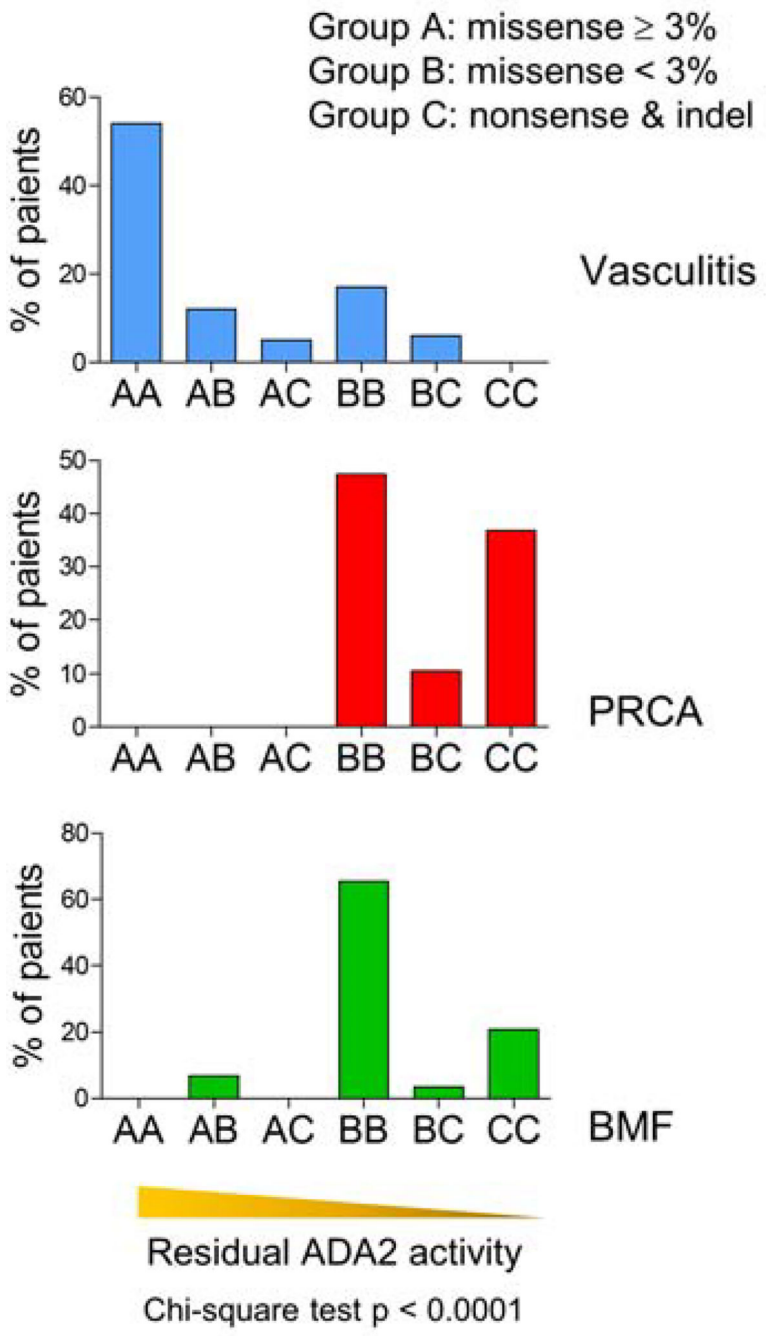


Figure 4. Genotype to phenotype analysis using patient mutation configurations.

Distribution of patients with vasculitis, PRCA or BMF phenotype in genotype categories assigned based on *ADA2* mutation type and residual enzymatic activity of missense mutations ($p < 0.0001$, Chi-square test). Bars represent the percentage of patients of the given phenotype.

Table 1.

Summary of clinical characteristics in DADA2 patients with PRCA or BMF

	PRCA	BMF
Number of cases	5	10
Median age of onset (year)	0.3	2.2
Sex (% female)	40	50
Anemia (%)	100	80
Lymphopenia (%)	0	40
Neutropenia (%)	0	90
Thrombocytopenia (%)	0	30
Low IgG (%)	20	30
Low IgM (%)	40	60
Low IgA (%)	60	50
Recurrent infection (%)	20	80
Stroke (%)	20	0
Skin vasculitis (%)	0	20
Oral ulcers / Gingivitis (%)	20	70
Hepatosplenomegaly (%)	40	90
Death (%)	20	50

Table 2.

Characterization of *ADA2* mutations

Protein	cDNA	n	Phenotype	Type	Domain	ADA2 activity (%WT)	Published phenotype [ref#]
G47W	c.139G>T	1	BMF	missense	Dimerization	0.3 ± 0.5	Vasculitis *[25]
R49Afs*13	c.137dupT	4	PRCA, BMF	frameshift	Dimerization	UD	Hemolytic anemia [8]
F178S	c.533T>C	2	BMF	missense	Catalytic	0.8 ± 0.6	PRCA [8]
F212del	c.634_636delTTC	1	BMF	deletion	Catalytic	0.8 ± 1.2	-
G321E	c.962G>A	1	PRCA	missense	Catalytic	1.8 ± 1.0	BMF [18]
G358R	c.1072G>A	4	BMF	missense	Catalytic	1.7 ± 0.6	PRCA [6]
K449Nfs*2	c.1346_1347insTT	1	BMF	frameshift	Catalytic	UD	-
K466Tfs*2	c.1397_1403AGGCTGAdel	1	BMF	frameshift	Catalytic	UD	PRCA [10]
V458D	c.1373T>A	1	BMF	missense	Catalytic	2.4 ± 0.4	BMF [13] Vasculitis *[26]

Abbreviations: UD, undetectable.

*This mutation was previously described in a patient with compound heterozygous mutations.